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PRIORITY DOCUMENT

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Modtaget

TITLE: Improved Bacillus Host Cell

TECHNICAL FIELD

Bacillus sp. are attractive hosts for the production of heterologous proteins due their ability to secrete proteins directly into the culture medium. They have a high capacity for protein secretion, are genetically highly amenable, nonpathogenic and free of endotoxins, and consequently a large variety of proteins from different organisms have been efficiently produced and secreted in Bacillus sp. i.e. in Bacillus licheniformis.

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Improved *Bacillus* host cells that provide better production economy, or better products e.g. in terms of stability, purity etc. are constantly in demand in the industry.

BACKGROUND

Industrial production in *Bacillus sp.* of products of interest such as heterologous polypeptides, amino acids, carbohydrates etc., even when such a product is secreted into the medium, very often requires a costly purification step of the product from the culture medium. Contaminant polypeptides native to the *Bacillus* production host cell are secreted into the culture medium, and they may have to be removed e.g. in order to ensure the stability of the product, or to obtain a sufficient purity of the product. Typically, the native secreted contaminant polypeptides could be proteolytic enzymes, nutrient uptake factors, signal molecules etc.

Naturally, it is of considerable interest to the industrial producers to reduce the costs associated with product purification steps, indeed it would be of commercial value if one or more purification steps could be completely eliminated.

25 **SUMMARY**

A problem to be solved by the present invention is how to reduce the necessary product purification required when producing products of interest in a *Bacillus licheniformis* host cell. The present invention provides a solution to the problem by reducing the amount of contaminant secreted native polypeptide(s) in the culture medium, this is achieved by reducing the expression of such polypeptide(s) in a mutated host cell. Production in a mutant host cell of the invention provides a culture medium with far fewer contaminants, and this in turn makes it much easier to purify the product of interest from the culture medium to the point where certain previously required steps may be completely eliminated from the production process. Production in a mutant host cell of the invention may also have a positive effect on the total product yield and shelf-life, since product stability is often hampered by the presence of contaminant polypeptides in the culture medium.

Accordingly, in a first aspect the invention relates to a *Bacillus licheniformis* mutant host cell derived from a parent *B. licheniformis* host cell, which mutant host cell is mutated in one or more gene(s) encoding one or more secreted polypeptide(s) which is at least 80% identical to one or more of the polypeptides shown in SEQ ID NO's: 2 to 200, preferably at least 85% identical, more preferably at least 90% identical, still more preferably at least 95% identical, and most preferably at least 97% identical to one or more of the polypeptides shown in SEQ ID NO's: 2 to 200, wherein the mutant host cell secretes at least 5% less of the one or more secreted polypeptide(s) than the parent host cell, when they are cultivated under comparable conditions.

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Preferably wherein the mutant host cell secretes at least 10% less, more preferably at least 20% less, still more preferably at least 30% less, even more preferably at least 40% less, yet more preferably at least 50% less, or at least 60% less, or at least 70% less, or at least 80%, or most preferably at least 90% less of the one or more secreted polypeptide(s) than the parent host cell, when they are cultivated under comparable conditions. Most preferably the mutant host cell secretes absolutely nothing of the one or more secreted polypeptide(s).

Comparable conditions of cultivation must be used in order to compare the secretion level of one or more secreted polypeptides in a mutant host cell of the invention with that in a parent host cell. They are cultivated separately under identical conditions in identical setups, of course allowing for the usual standard deviations of the operating parameters normally associated with growth experiments, such as temperature control etc. The quantification of the expression level of one or more secreted polypeptide(s) is done by standard text-book assay techniques as known in the art, often based on the biological activity of the one or more secreted polypeptide(s) *i.e.* if a secreted polypeptide is an amylase, then an amylase-activity based quantification assay is used. To quantify a secreted polypeptide of unknown activity, immuno based or mass-spec based assays may be used.

In a second aspect the invention relates to a process for producing at least one product of interest in a *Bacillus licheniformis* mutant host cell, comprising cultivating a *B.licheniformis* mutant host cell as defined in the previous aspect in a suitable medium, whereby the said product is produced.

Finally, an aspect of the invention relates to a use of a *Bacillus licheniformis* mutant host cell as defined in the first aspect for producing at least one product of interest comprising cultivating the mutant host cell in a suitable medium whereby the said product is produced.

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DEFINITIONS

Nucleic acid construct: When used herein, the term "nucleic acid construct" means a nucleic acid molecule, either single- or double-stranded, which is isolated from a naturally occurring gene or which has been modified to contain segments of nucleic acids in a manner that would not otherwise exist in nature. The term nucleic acid construct is synonymous with the term "expression cassette" when the nucleic acid construct contains the control sequences required for expression of a coding sequence of the present invention.

Control sequence: The term "control sequences" is defined herein to include all components, which are necessary or advantageous for the expression of a polypeptide of the present invention. Each control sequence may be native or foreign to the nucleotide sequence encoding the polypeptide. Such control sequences include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, promoter, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the nucleotide sequence encoding a polypeptide.

Operably linked: The term "operably linked" is defined herein as a configuration in which a control sequence is appropriately placed at a position relative to the coding sequence of the DNA sequence such that the control sequence directs the expression of a polypeptide.

Coding sequence: When used herein the term "coding sequence" is intended to cover a nucleotide sequence, which directly specifies the amino acid sequence of its protein product. The boundaries of the coding sequence are generally determined by an open reading frame, which usually begins with the ATG start codon. The coding sequence typically include DNA, cDNA, and recombinant nucleotide sequences.

Expression: In the present context, the term "expression" includes any step involved in the production of the polypeptide including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification, and secretion.

Expression vector: In the present context, the term "expression vector" covers a DNA molecule, linear or circular, that comprises a segment encoding a polypeptide of the invention, and which is operably linked to additional segments that provide for its transcription.

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DETAILED DISCLOSURE

A Bacillus licheniformis mutant host cell derived from a parent B. licheniformis host cell, which mutant host cell is mutated in one or more gene(s) encoding one or more secreted polypeptide(s) which is at least 80% identical to one or more of the polypeptides shown in SEQ ID NO's: 2 to 200, wherein the mutant host cell secretes at least 5% less of the one or more secreted polypeptide(s) than the parent host cell, when they are cultivated under comparable conditions.

The term "parent host cell" in the context of the present invention means a cell which is genetically identical, or isogenic, to the progeny mutant or mutant cell of the present invention, except for the mutated one or more gene(s) encoding one or more secreted polypeptide(s) in said mutant.

The degree of identity, or %-identity of polypeptide sequences can suitably be investigated by aligning the sequences using a computer program known in the art, such as "GAP" provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711)(Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453). Using GAP with the following settings for DNA sequence comparison: GAP creation penalty of 5.0 and GAP extension penalty of 0.3".

The object of the present invention is to provide a cleaner culture medium so as to reduce the product purification to a minimum, and this may be done according to the invention by reducing or even completely abolishing the expression of genes expressing native secreted polypeptides via mutagenisation of those genes. One of the very well-known method of ensuring that a gene is not expressed into an active polypeptide within a cell is simply to delete or partially delete the encoding gene. Many techniques have been described in the art on how to specifically delete or partially delete one or more gene(s) in the genome of a cell, and certainly from the genome of a *Bacillus Iicheniformis* cell (see e.g. Novozymes A/S WO 01/90393, Novozymes A/S WO 02/00907, and Example 1 herein). Accordingly, a preferred embodiment of the present invention relates to a host cell of the first aspect, which is mutated by a partial or complete deletion of the one or more gene(s) encoding the one or more secreted polypeptide(s).

A specific example of such a deletion or partial deletion is shown in an example herein, where a gene encoding the native secreted polypeptide shown in SEQ ID NO: 134 is deleted from a *Bacillus licheniformis* host cell. So, a preferred embodiment of the present invention

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relates to a host cell of the first aspect, which is mutated by a partial or complete deletion of a gene encoding a secreted polypeptide which is at least 80% identical to the polypeptide shown in SEQ ID NO: 134, more preferably at least 85%, still more preferably at least 90%, even more preferably at least 95%, and most preferably at least 97% identical to the polypeptide shown in SEQ ID NO: 134.

As already mentioned, it is an object of the invention to provide a cleaner culture medium, and the more secreted contaminant polypeptides that are eliminated, the fewer will have to be removed in a subsequent product purification. A preferred embodiment of the present invention relates to a host cell of the first aspect, which is mutated in two or more genes encoding two or more secreted polypeptides.

The product of interest to be produced by the mutant host cell of the first aspect may be one or more polypeptide(s) encoded by one or more heterologous gene(s). Consequently, a preferred embodiment of the present invention relates to a host cell of the first aspect, which comprises one or more heterologous gene(s) encoding one or more heterologous polypeptide(s).

In the industrial production of polypeptides it is of interest to achieve a product yield as high as possible. One way to increase the yield is to increase the copy number of a gene encoding a polypeptide of interest. This can be done by placing the gene on a high copy number plasmid. However, plasmids are unstable and are often lost from the host cells if there is no selective pressure during the cultivation of the host cells. Another way to increase the copy number of the gene of interest is to integrate it into the host cell chromosome in multiple copies. Integration of two genes has been described in WO 91/09129 and WO 94/14968 (Novozymes A/S) the content of which is hereby incorporated by reference. A preferred embodiment of the present invention relates to a host cell of the first aspect, wherein the heterologous gene(s) is present in at least two copies, preferably at least 4 copies, and most preferably at least 6 copies. In another embodiment the heterologous gene(s) is present in at least ten coples. If carried on a plasmid the gene(s) may be present in several hundred copies per cell, so in a still further embodiment of the present invention the heterologous gene(s) is present in at least 100 copies.

Integration of two genes closely spaced in anti-parallel tandem to achieve better stability has been described in WO 99/41358 (Novozymes A/S) the content of which is hereby incorporated by reference, as well as the stable chromosomal multi-copy integration of genes described in WO 02/00907 (Novozymes A/S) the content of which is incorporated herein by

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reference. A preferred embodiment of the present invention relates to a host cell of the first aspect, wherein the heterologous gene(s) are stably integrated into the genome of the cell.

Selection of chromosomal integrant has for convenience resulted in the use of selectable markers such as antibiotic resistance markers. However it is desirable if possible to avoid the use of antibiotic marker genes. WO 01/90393 discloses a method for the integration of a gene in the chromosome of a host cell without leaving antibiotic resistance markers behind in the strain, the content of which is hereby incorporated by reference A preferred embodiment of the present invention relates to a host cell of the first aspect wherein the heterologous gene(s) is integrated into the genome of the cell without leaving any antibiotic resistance marker genes at the site of integration.

The present invention also relates to nucleic acid constructs comprising a nucleotide sequence encoding a product of interest, which may be operably linked to one or more control sequences that direct the expression of the coding sequence in a suitable host cell under conditions compatible with the control sequences.

A nucleotide sequence encoding a polypeptide ofinterest may be manipulated in a variety of ways to provide for expression of the polypeptide. Manipulation of the nucleotide sequence prior to its insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying nucleotide sequences utilizing recombinant DNA methods are well known in the art.

Other ways of increasing the product yield would be to increase promoter activity of the specific promoter regulating the expression of a specific gene of interest. Also a more general increase in the activity of several promoters at the same time could lead to an improved product yield. The control sequence may be an appropriate promoter sequence, a nucleotide sequence which is recognized by a host cell for expression of the nucleotide sequence. The promoter sequence contains transcriptional control sequences, which mediate the expression of the polypeptide. The promoter may be any nucleotide sequence which shows transcriptional activity in the host cell of choice including mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the host cell.

Examples of suitable promoters for directing the transcription of the nucleic acid constructs of the present invention, especially in a bacterial host cell, are the promoters obtained from the E. coli lac operon, Streptomyces coelicolor agarase gene (dagA), Bacillus subtilis

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levansucrase gene (sacB), Bacillus licheniformis alpha-amylase gene (amyL), Bacillus stearothermophilus maltogenic amylase gene (amyM), Bacillus amyloliquefaciens alpha-amylase gene (amyQ), Bacillus licheniformis penicillinase gene (penP), Bacillus subtilis xylA and xylB genes, and prokaryotic beta-lactamase gene (Villa-Kamaroff et al., 1978, Proceedings of the National Academy of Sciences USA 75: 3727-3731), as well as the tac promoter (DeBoer et al., 1983, Proceedings of the National Academy of Sciences USA 80: 21-25). Further promoters are described in "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242: 74-94; and in Sambrook et al., 1989, supra.

Other useful promoters are described in WO 93/10249, WO 98/07846, and WO 99/43835 (Novozymes A/S) the contents of which are incorporated fully herein by reference. A preferred embodiment of the present invention relates to a host cell of the first aspect, wherein the heterologous gene(s) are transcribed from a heterologous promoter or from an artificial promoter.

The control sequence may also be a suitable transcription terminator sequence, a sequence recognized by a host cell to terminate transcription. The terminator sequence is operably linked to the 3' terminus of the nucleotide sequence encoding the polypeptide. Any terminator which is functional in the host cell of choice may be used in the present invention.

The control sequence may also be a suitable leader sequence, a nontranslated region of an mRNA which is important for translation by the host cell. The leader sequence is operably linked to the 5' terminus of the nucleotide sequence encoding the polypeptide. Any leader sequence that is functional in the host cell of choice may be used in the present invention.

The control sequence may also be a polyadenylation sequence, a sequence operably linked to the 3' terminus of the nucleotide sequence and which, when transcribed, is recognized by the host cell as a signal to add polyadenosine residues to transcribed mRNA. Any polyadenylation sequence which is functional in the host cell of choice may be used in the present invention.

The control sequence may also be a signal peptide coding region that codes for an amino acid sequence linked to the amino terminus of a polypeptide and directs the encoded polypeptide into the cell's secretory pathway. The 5' end of the coding sequence of the nucleotide sequence may inherently contain a signal peptide coding region naturally linked in translation reading frame with the segment of the coding region which encodes the secreted polypeptide. Alternatively, the 5' end of the coding sequence may contain a signal peptide

coding region which is foreign to the coding sequence. The foreign signal peptide coding region may be required where the coding sequence does not naturally contain a signal peptide coding region. Alternatively, the foreign signal peptide coding region may simply replace the natural signal peptide coding region in order to enhance secretion of the polypeptide. However, any signal peptide coding region which directs the expressed polypeptide into the secretory pathway of a host cell of choice may be used in the present invention.

Effective signal peptide coding regions for bacterial host cells are the signal peptide coding regions obtained from the genes for Bacillus NCIB 11837 maltogenic amylase, Bacillus stearothermophilus alpha-amylase, Bacillus licheniformis subtilisin, Bacillus licheniformis beta-lactamase, Bacillus stearothermophilus neutral proteases (nprT, nprS, nprM), and Bacillus subtilis prsA. Further signal peptides are described by Simonen and Palva, 1993, Microbiological Reviews 57: 109-137.

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The control sequence may also be a propeptide coding region that codes for an amino acid sequence positioned at the amino terminus of a polypeptide. The resultant polypeptide is known as a proenzyme or propolypeptide (or a zymogen in some cases). A propolypeptide is generally inactive and can be converted to a mature active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide. The propeptide coding region may be obtained from the genes for Bacillus subtilis alkaline protease (aprE), Bacillus subtilis neutral protease (nprT), Saccharomyces cerevisiae alpha-factor, Rhizomucor miehei aspartic proteinase, and Myceliophthora thermophila laccase (WO 95/33836).

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Where both signal peptide and propeptide regions are present at the amino terminus of a polypeptide, the propeptide region is positioned next to the amino terminus of a polypeptide and the signal peptide region is positioned next to the amino terminus of the propeptide region.

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It may also be desirable to add regulatory sequences which allow the regulation of the expression of the polypeptide relative to the growth of the host cell. Examples of regulatory systems are those which cause the expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Regulatory systems in prokaryotic systems include the lac, tac, and trp operator systems. In yeast, the ADH2 system or GAL1 system may be used. In eukaryotic systems, these include the dihydrofolate reductase gene which is amplified in the presence of methotrexate, and the

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metallothionein genes which are amplified with heavy metals. In these cases, the nucleotide sequence encoding the polypeptide would be operably linked with the regulatory sequence.

The present invention also relates to recombinant expression vectors comprising the nucleic acid construct of the invention. The various nucleotide and control sequences described above may be joined together to produce a recombinant expression vector which may include one or more convenient restriction sites to allow for insertion or substitution of the nucleotide sequence encoding the polypeptide at such sites. Alternatively, the nucleotide sequence of the present invention may be expressed by inserting the nucleotide sequence or a nucleic acid construct comprising the sequence into an appropriate vector for expression. In creating the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression.

The recombinant expression vector may be any vector (e.g., a plasmid or virus) which can be conveniently subjected to recombinant DNA procedures and can bring about the expression of the nucleotide sequence. The choice of the vector will typically depend on the compatibility of the vector with the host cell into which the vector is to be introduced. The vectors may be linear or closed circular plasmids.

The vector may be an autonomously replicating vector, i.e., a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome.

The vector may contain any means for assuring self-replication. Alternatively, the vector may be one which, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Furthermore, a single vector or plasmid or two or more vectors or plasmids which together contain the total DNA to be introduced into the genome of the host cell, or a transposon may be used.

The vectors of the present invention preferably contain one or more selectable markers which permit easy selection of transformed cells. A selectable marker is a gene the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like.

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Examples of bacterial selectable markers are the dal genes from Bacillus subtilis or Bacillus licheniformis, or markers which confer antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracycline resistance.

The vectors of the present invention preferably contain an element(s) that permits stable integration of the vector into the host cell's genome or autonomous replication of the vector in the cell independent of the genome.

For integration into the host cell genome, the vector may rely on the nucleotide sequence encoding the polypeptide or any other element of the vector for stable integration of the vector into the genome by homologous or nonhomologous recombination. Alternatively, the vector may contain additional nucleotide sequences for directing integration by homologous recombination into the genome of the host cell. The additional nucleotide sequences enable the vector to be integrated into the host cell genome at a precise location(s) in the To increase the likelihood of integration at a precise location, the chromosome(s). integrational elements should preferably contain a sufficient number of nucleotides, such as 100 to 1,500 base pairs, preferably 400 to 1,500 base pairs, and most preferably 800 to 1,500 base pairs, which are highly homologous with the corresponding target sequence to enhance the probability of homologous recombination. The integrational elements may be any sequence that is homologous with the target sequence in the genome of the host cell. Furthermore, the integrational elements may be non-encoding or encoding nucleotide sequences. On the other hand, the vector may be integrated into the genome of the host cell by non-homologous recombination.

For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host cell in question. Examples of bacterial origins of replication are the origins of replication of plasmids pBR322, pUC19, pACYC177, and pACYC184 permitting replication in E. coli, and pUB110, pE194, pTA1060, and pAMß1 permitting replication in Bacillus. The origin of replication may be one having a mutation which makes its functioning temperature-sensitive in the host cell (see, e.g., Ehrlich, 1978, Proceedings of the National Academy of Sciences USA 75: 1433).

More than one copy of a nucleotide sequence of the present invention may be inserted into the host cell to increase production of the gene product. An increase in the copy number of the nucleotide sequence can be obtained by integrating at least one additional copy of the sequence into the host cell genome or by including an amplifiable selectable marker gene with the nucleotide sequence where cells containing amplified copies of the selectable

marker gene, and thereby additional copies of the nucleotide sequence, can be selected for by cultivating the cells in the presence of the appropriate selectable agent.

The procedures used to ligate the elements described above to construct the recombinant expression vectors of the present invention are well known to one skilled in the art (see, e.g., Sambrook et al., 1989, supra).

The introduction of a vector into a bacterial host cell may, for instance, be effected by protoplast transformation (see, e.g., Chang and Cohen, 1979, Molecular General Genetics 168: 111-115), using competent cells (see, e.g., Young and Spizizin, 1961, Journal of Bacteriology 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, Journal of Molecular Biology 56: 209-221), electroporation (see, e.g., Shigekawa and Dower, 1988, Biotechniques 6: 742-751), or conjugation (see, e.g., Koehler and Thorne, 1987, Journal of Bacteriology 169: 5771-5278).

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A preferred embodiment of the present invention relates to a host cell of the first aspect, wherein the heterologous gene(s) are comprised in an operon, preferably a polycistronic operon. The term "operon" in the context of the present invention means a polynucleotide comprising several genes that are clustered and perhaps even transcribed together into a polycistronic mRNA, e.g. genes coding for the enzymes of a metabolic pathway. The transcription of an operon may be initiated at a promoter region and controlled by a neighboring regulatory gene, which encodes a regulatory protein, which in turn binds to the operator sequence in the operon to respectively inhibit or enhance the transcription. The gene or the operon can be carried on a suitable plasmid that can be stably maintained, e.g. capable of stable autonomous replication in the host cell (the choice of plasmid will typically depend on the compatibility of the plasmid with the host cell into which the plasmid is to be introduced) or it can be carried on the chromosome of the host. The said gene may be endogenous to the host cell in which case the product of interest is a protein naturally produced by the host cell and in most cases the gene will be in it normal position on the chromosome. If the gene encoding the product of interest is an exogenous gene, the gene could either be carried on a suitable plasmid or it could be integrated on the host chromosome. In one embodiment of the invention the eubacterium is a recombinant eubacterium. Also the product of interest may in another embodiment be a recombinant protein.

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The product of interest is any gene product or product of a metabolic pathway which is industrially useful and which can be produced in a bacterial cell such as a *B. licheniformis*.

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In one preferred embodiment, the heterologous polypeptide(s) is an antimicrobial peptide, or a fusion peptide comprising a peptide part which in its native form has antimicrobial activity.

In another preferred embodiment, the heterologous polypeptide(s) has biosynthetic activity and produces a compound or an intermediate of interest.

Yet another embodiment relates to a host cell of the first aspect, wherein the compound or intermediate of interest comprises vitamins, amino acids, antibiotics, carbohydrates, or surfactants, and preferably the carbohydrates comprise hyaluronic acid.

In one embodiment the heterologous polypeptide(s) is an enzyme, particularly the enzyme is an enzyme of a class selected from the group of enzyme classes consisting of oxidoreductases (EC 1), transferases (EC 2), hydrolases (EC 3), lyases (EC 4), isomerases (EC 5), and ligases (EC 6). Preferably the enzyme is an enzyme with an activity selected from the group consisting of aminopeptidase, amylase, amyloglucosidase, mannanase, carbohydrase, carboxypeptidase, catalase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, galactosidase, beta-galactosidase, glucoamylase, glucose oxidase, glucosidase, haloperoxidase, hemicellulase, invertase, isomerase, laccase, ligase, lipase, lyase, mannosidase, oxidase, pectinase, peroxidase, phytase, phenoloxidase, polyphenoloxidase, protease, ribonuclease, transferase, transglutaminase, or xylanase. Preferably the enzyme is an amylase or a mannanase.

A second aspect of the invention relates to a process for producing at least one product of interest in a *Bacillus licheniformis* mutant host cell, comprising cultivating a *B.licheniformis* mutant host cell as defined in the first aspect of the invention in a suitable medium, whereby the said product is produced. One embodiment relates to a process of the second aspect, further comprising isolating or purifying the product of interest. Suitable media for the cultivation is described below as well as methods for the purification or isolation of the produced product which is an optional additional step to the process of the present invention.

In the production methods of the present invention, the cells are cultivated in a nutrient medium suitable for production of the polypeptide using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a

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suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (e.g., in catalogues of the American Type Culture Collection). If the polypeptide is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide is not secreted, it can be recovered from cell lysates.

The medium used to culture the cells may be any conventional medium suitable for growing the host cells, such as minimal or complex media containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. in catalogues of the American Type Culture Collection). The media are prepared using procedures known in the art (see, e.g., references for bacteria and yeast; Bennett, J.W. and LaSure, L., editors, *More Gene Manipulations in Fungi*, Academic Press, CA, 1991).

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The polypeptides may be detected using methods known in the art that are specific for the polypeptides. These detection methods may include use of specific antibodies, formation of an enzyme product, or disappearance of an enzyme substrate. For example, an enzyme assay may be used to determine the activity of the polypeptide as described herein.

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The resulting polypeptide may be recovered by methods known in the art. For example, the polypeptide may be recovered from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray-drying, evaporation, or precipitation.

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The polypeptides of the present invention may be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing), differential solubility (e.g., ammonium sulfate precipitation), SDS-PAGE, or extraction (see, e.g., *Protein Purification*, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989).

A third aspect of the present invention relates to the use of a *Bacillus licheniformis* mutant host cell as defined in the first aspect for producing at least one product of interest comprising cultivating the mutant host cell in a suitable medium whereby the said product is produced, and optionally isolating or purifying the produced product.

The present invention is further illustrated by the following examples, which, however, are not to be construed as limiting the scope of protection. The features disclosed in the foregoing description and in the following examples may, both separately and in any combination thereof, be material for realising the invention in diverse forms thereof.

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EXAMPLES

Example 1

The gene encoding a small extracellular protein from *B. licheniformis* is included in the sequence shown in SEQ ID NO: 133, where the start codon of the protein encoding sequence is the ATG in position 601, and the stopcodon is the TAA in position 979.

A vector designed to allow deletion of the entire open reading frame is constructed as follows:

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1) An upstream DNA fragment is prepared by PCR amplification using chromosomal *B. licheniformis* DNA as template and the following primers:

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ECORI <L12 574-594> (SEQ ID NO: 201)

5'-gactgaattcgtgcgagttcctccacattcg-3'
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HindIII BglII<L12 1074-1052> (SEQ ID NO: 202) 5'-gactaagcttagatctactctataagttagtttgtcacc-3'
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The amplified fragment is digested with EcoRI og HindIII, inserted between the EcoRI and HindIII sites in pUC19, and the ligation mixture transformed into *E. coli* selecting ampicillin resistance (200 microg/ml).

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2) The cloned DNA fragment is excised as an EcoRi-BgIII fragment, and ligated to the 5.1 kb EcoRi-BgIII fragment of pSJ2739 (Described in U.S. Patent 6,100,063, Fig. 10). The ligation mixture is transformed into *B. subtilis* DN1885 (Diderichsen et al., 1990 (Diderichsen, B., Wedsted, U., Hedegaard, L., Jensen, B. R., Sjøholm, C. (1990). Cloning of aldB, which encodes α-acetolactate decarboxylase, an exoenzyme from Bacillus brevis. J. Bacteriol. 172, 4315-4321)), selecting erythromycln resistance (5 microg/ml) at 30 °C.

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3) A downstream DNA fragment is prepared by PCR amplification using chromosomal *B. licheniformis* DNA as template and the following primers:

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KpnI BglII BamHI<L12 1500-1520> (SEQ ID NO: 203)
5'-gactggtaccagatctggatccgaaaacggttgctgtcaacgg-3'

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The amplified fragmentet is digested with KpnI og HindIII, and inserted into KpnI + HindIII digested pUC19. Transformation is into *E. coli*.

- 4) A DNA fragment containing a spectinomycin resistance gene (*spc*) flanked by resolvase sites (*res*) originating from plasmid pAMβ1 is excised as a 1.5 kb Bcll-BamHI fragment from plasmid pSJ3358 (described in U.S.Patent 5,882,888), and inserted into the BamHI site of the plasmid constructed above, under 3). Transformation is into *E. coli*.
- 5) The entire "res-spc-res-downstream DNA fragment" segment is excised from the plasmid prepared under 4), above, using enzymes BgllI and HindIII, and is inserted in the plasmid prepared under 2), above, which has been digested with BgllI and HindIII. Transformation is into B. subtilis DN1885, selecting spectinomycin resistance (120 μ g/ml) and erythromycin resistance (5 μ g/ml) at 30 °C.
- 6) The plasmid constructed under 5), above, is transformed into donor strain PP289-5 (described in US 5,882,888) for easy transfer into *B. licheniformis* by conjugation.
- B. licheniformis strains, which do not produce the small extracellular protein, are constructed by the following procedure:
- The plasmid constructed under 5), above, is transferred into the *B. licheniformis* strain by conjugation from the *B. subtilis* donor strain constructed under 6), above, as described in US5,882,888. Strains, in which the plasmid has integrated into the chromosome, are selected by isolation of colonies able to grow at 50 °C on plates containing erythromycin. Such colonies are then inoculated into liquid medium without antibiotics, and propagated overnight at 30 °C. These cultures are used to inoculate further liquid cultures, without antibiotics, again propagated overnight at 30 °C. If needed, this is repeated one or more

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times. Aliquots from each overnight culture are spread on plates with spectinomycin (120 μ g/ml) and incubated overnight at 30 °C, then replica plated onto plates with erythromycin (5 μ g/ml). Colonies able to grow on spectinomycin, but sensitive to erythromycin, are picked and further investigated, e.g. by southern analysis and growth experiments. Such colonies will have the chromosomal gene encoding the small extracellular protein replaced by the *resspe-res* cassette.

The spectinomycin resistance gene may subsequently be deleted from the strain by introduction of a plasmid expressing the pAM β 1 resolvase gene, as described in US 5,882,888.

Alternatively, the *res-spc-res* cassette may be deleted in its entirety using a plasmid containing just the joined upstream and downstream regions flanking the gene for the extracellular protein, or such a plasmid may be used directly in the first step to delete the gene for the extracellular protein in the *B. licheniformis* strain.

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CLAIMS

- 1. A Bacillus licheniformis mutant host cell derived from a parent B. licheniformis host cell, which mutant host cell is mutated in one or more gene(s) encoding one or more secreted polypeptide(s) which is at least 80% identical to one or more of the polypeptides shown in SEQ ID NO's: 2 to 200, wherein the mutant host cell secretes at least 5% less of the one or more secreted polypeptide(s) than the parent host cell, when they are cultivated under comparable conditions.
- 2. The host cell according to claim 1, which is mutated by a partial or complete deletion of the one or more gene(s) encoding the one or more secreted polypeptide(s).
 - 3. The host cell according to claim 1 or 2, which is mutated by a partial or complete deletion of a gene encoding a secreted polypeptide which is at least 80% identical to the polypeptide shown in SEQ ID NO: 134.
 - 4. The host cell according to any of claims 1 3, which is mutated in two or more genes encoding two or more secreted polypeptides.
- 5. The host cell according to any of claims 1 4, which comprises one or more heterologous gene(s) encoding one or more heterologous polypeptide(s).
 - 6. The host cell according to claim 5, wherein the heterologous gene(s) is present in at least two copies.
- 7. The host cell according to claim 5 or 6, wherein the heterologous gene(s) are stably integrated into the genome of the cell.
 - 8. The host cell according to any of claims 5 7, wherein the heterologous gene(s) is integrated into the genome of the cell without leaving any antibiotic resistance marker genes at the site of integration.
 - 9. The host cell according to any of claims 5 8, wherein the heterologous gene(s) are transcribed from a heterologous promoter or from an artificial promoter.
- 35 10. The host cell according to any of claim 5 9, wherein the heterologous gene(s) are comprised in an operon, preferably a polycistronic operon.

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- 11. The host cell according to any of claims 5 10, wherein the heterologous polypeptide(s) is an antimicrobial peptide, or a fusion peptide comprising a peptide part which in its native form has antimicrobial activity.
- 12. The host cell according to any of claims 5 10, wherein the heterologous polypeptide(s) has biosynthetic activity and produces a compound or an intermediate of interest.
 - 13. The host cell according to claim 12, wherein the compound or intermediate of interest comprises vitamins, amino acids, antibiotics, carbohydrates, or surfactants.
 - 14. The host cell according to claim 13, wherein the carbohydrates comprise hyaluronic acid.
 - 15. The host cell according to any of claims 5 10, wherein the heterologous polypeptide(s) is an enzyme, preferably a secreted enzyme.
 - 16. The host cell according to claim 15, wherein the enzyme is is an enzyme of a class selected from the group of enzyme classes consisting of oxidoreductases (EC 1), transferases (EC 2), hydrolases (EC 3), lyases (EC 4), isomerases (EC 5), and ligases (EC 6).
 - 17. The host cell according to claim 16, wherein the enzyme is an enzyme with an activity selected from the group of enzyme activities consisting of aminopeptidase, amylase, amyloglucosidase, mannanase, carbohydrase, carboxypeptidase, catalase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, galactosidase, beta-galactosidase, glucoamylase, glucose oxidase, glucosidase, haloperoxidase, hemicellulase, invertase, isomerase, laccase, ligase, lipase, lyase, mannosidase, oxidase, pectinase, peroxidase, phytase, phenoloxidase, polyphenoloxidase, protease, ribonuclease, transferase, transglutaminase, and xylanase.
 - 18. The host cell according to claim 17, wherein the enzyme is an amylase or a mannanase.
 - 19. A process for producing at least one product of interest in a *Bacillus licheniformis* mutant host cell, comprising cultivating a *B.licheniformis* mutant host cell as defined in any of the claims 1 18 in a suitable medium, whereby the said product is produced.
 - 20. The process according to claim 19, further comprising isolating or purifying the product of interest.

- 21. A use of a *Bacillus licheniformis* mutant host cell as definde in any of the claims 1 18 for producing at least one product of interest comprising cultivating the mutant host cell in a suitable medium whereby the said product is produced.
- 22. The use according to claim 21 further comprising isolating or purifying the product of interest.

ABSTRACT

TITLE: Improved Bacillus Host Cell.

A *Bacillus licheniformis* mutant host cell derived from a parent *B. licheniformis* host cell, which mutant host cell is mutated in one or more gene(s) encoding one or more secreted polypeptide(s) which is at least 80% identical to one or more of the polypeptides shown in SEQ ID NO's: 2 to 200, wherein the mutant host cell secretes at least 5% less of the one or more secreted polypeptide(s) than the parent host cell, when they are cultivated under comparable conditions.

Patent- og Varemærkestyrelsen

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	Clausen, Ib Groth
	Jørgensen, Steen Troels
	Olsen, Peter Bjarke
	Rasmussen, Michael Dolberg

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Page 10

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Leu Thr Asn Asp Leu Leu Thr Leu Tyr Gly Ala Lys Asp Ser Ala Glu 50 60

Leu Thr Tyr Gln Ile Pro Ala Gly Ala Ser Ser Thr His Gln Gln Leu 65 70 75

Thr Leu Lys Tyr Glu Ala Ser Asp Leu Leu Ile Ser Pro Ser Ser Leu 85 90 95

Thr Ala Glu Ile Asp Gly Glu Pro Val Lys Thr Val Lys Leu Glu Gly 100 105 110

Asn Asn Gly Lys Lys Thr Leu Lys Leu Ser Leu Asn Lys Ser Gln Ser 115 120

Ser Pro Gly Phe His Ser Leu Ser Leu Lys Phe Tyr Gly Val Val His 130 135 140

Glu Gly Val Cys Val Arg Gln Asp Ser Ser Gly Asn Trp Ile Lys Ile 145 150 155 160 Page 29

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		gct Ala															680	
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Page 44

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g V	tt al	gca Ala	cta Leu 480	aac Asn	gga Gly	ggc Gly	atc Ile	ctt Leu 485	aag Lys	gaa Glu	gat Asp	gcg Ala	ccg Pro 490	gga Gly	aaa Lys	ctg Leu	2330
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Asn Ser Ile Gln Leu Lys Val Glu Gln 585

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gaa ctt gaa cac gat ccc gac gtc ctg tat gtc gaa gac aac ctc ccg Glu Leu Glu His Asp Pro Asp Val Leu Tyr Val Glu Asp Asn Leu Pro 80 85 90	773
gta gct gct gcc gac agc acc gct cta aaa gct ttc tcc agc agc aca Page 54	821

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140 145 150 155

· 869

917

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Ile Asp Trp Ser Ile Gln His Gly Ile Asp Ile Ile Asn Met Ser Leu
220 235

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Tyr Lys Arg Gly Ile Ile Leu Val Gly Ala Ser Gly Asn Ala Gly Asn
255 260 265

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270 275 280

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335
340
345

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350 355 360

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Ile Glu Gln Ala Asp Thr Val Glu His Val Tyr Arg His Ile Pro Ala 50 60

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Pro Asp Val Leu Tyr Val Glu Asp Asn Leu Pro Val Ala Ala Asp 90 95

Ser Thr Ala Leu Lys Ala Phe Ser Ser Ser Thr Ala Gln Asn Ala Ser 100 105 110

Ala Phe Ser Gln Trp Asn Ile Lys Leu Ile Gln Ala Ala Leu Ala Trp 115 120

Asn Lys Gly Leu Thr Gly Lys Gln Val Lys Ile Ala Val Ile Asp Ser 130 140

Gly Ile Ser Pro His Glu Glu Leu Ser Ile Ala Gly Gly Ala Ser Met 145 150 160

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Page 60

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2620

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Asp Val Ala Leu Glu Val Ser Leu Arg Ile Arg Asp Tyr Leu Gln Glu 70 75

Gln Gly Ala Leu Val Met Leu Thr Arg Glu Asp Asp His Asp Leu Ala 85 90

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Arg Ile His Gly Ile Tyr Leu Met Gln Asn Val Lys Lys Pro Gly Ala 180 185 190

Leu Val Glu Ile Gly Phe Leu Ser Asn Pro Glu Glu Ala Lys Gln Leu 195 200 205

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773

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Page 100

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<213> Bacillus licheniformis

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Gly Gly Asp Gly Arg Ala Ser Ala Asp Ser Asp Lys Gly Tyr Gly Arg 50 60

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Leu Cys Lys Asn Val Gly Asn Asp Gln Gly Val Lys Ala Ser Gly Arg 115 120 125

Asn Val Ser Arg Ser Ala Gln Val Ser Ala Val Ser Lys Ser Arg Ile 130 140

Gln Glu Leu Ile His Ala Leu Pro Leu Lys Ala Arg Thr Glu Arg Gly 145 150 160

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Gln val Glu Thr Val Glu Thr Ala Lys Ser Asp Leu His Thr Pro Ile 210 215 220

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Arg Ala Tyr Tyr Gly Gly Ser Lys Lys Phe Phe Lys Glu Glu Ser Ser 290 295 Lys Leu Leu Gln Gly Ala Asn Lys Lys Asn Ala Ser Leu Ala Asn Gly 305 310 320 Ala Leu Gly Ile Ile Glu Leu Asn Asp Tyr Thr Leu Lys Lys Val Met Lys Pro Leu Ile Ala Ser Asn Thr Val Thr Asp Glu Ile Glu Arg 340 350 Ala Asn Leu Phe Lys Met Asn Gly Lys Trp Tyr Leu Phe Thr Asp Ser Arg Gly Ser Lys Met Thr Ile Asp Gly Ile Gly Ser Lys Asp Ile Tyr 370 380 Met Leu Gly Tyr Val Ser Gly Ser Leu Thr Gly Pro Phe Lys Pro Leu 385 390 395 Asn Lys Ser Gly Leu Val Leu His Met Asp Gln Asp Tyr Asn Asp Ile 405 410 Thr Phe Thr Tyr Ser His Phe Ala Val Pro Gln Lys Lys Gly Asp Glu
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Tyr Ser Leu Tyr Leu Phe Glu Gly Leu Lys Ser Ser Gly Asn Thr Asp 115 120 125

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Lys Ala Ile Gln Glu Ile Leu Asn Asp Asp Lys Leu Asn Glu Thr Leu 50 60

Val Met Asp Glu Lys Thr Val Lys Glu Thr Val Glu Lys Thr Met Thr 65 70 75 80

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Phe Ala Glu Gly Phe Ala Lys Thr Leu Gln Asn Glu His Glu Lys Val 100 105 110

Leu Lys Lys Leu Met Lys Asp Pro Glu Tyr Gln Lys Met Leu Met Gln 115 120 125

Val Met Gln Asp Pro Glu Met Ala Lys Lys Tyr Gly Glu Leu Val Arg 130 135 140

Ser Gln Glu Phe Arg Ser His Leu Gln Glu Val Ile Ser Asp Thr Leu 145 150 160

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<210> 68

<211> 401

<212> PRT

<213> Bacillus licheniformis

<400> 68

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<211> **1**547

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<213> Bacillus licheniformis

<220>

<221> CDS

<222> (501)..(1046)

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cgct	cccg	gg a	ggtc	ccaa	a aa	aatt	tttt	tgc	aaaa	aaa	aatt	tttc	ככ כ	ataa	ggctc	300
tagt	gtta	tg a	gaaa	aaaa	t cc	ggga	acgg	aat	caag	gac	cata	aaaa	tt t	tttc	tggcc	360
aacc	caaa	ac c	ccgg	tgcg	t tt	aagt	cgtc	ata	aata	aga	aacc	agcg	ga g	gaaa	aattt	420
ttct	cgca	ac c	ctct	tgta	a to	tato	tgac	gtt	attg	taa	catt	tgta	at a	taag	agata	480
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tta Leu	ctg Leu	atc Ile	ttg Leu 15	cct Pro	gcc Ala	gga Gly	gcg Ala	tcc Ser 20	ctc Leu	gca Ala	gcg Ala	aaa Lys	aat Asn 25	caa Gln	aca Thr	581
tca ser	ggg G1y	aat Asn 30	tta Leu	aca Thr	aat Asn	aag Lys	caa G1n 35	gtc Val	atg Met	caa Gln	tta Leu	acc Thr 40	ttg Leu	cag Gln	gca Ala	629
c g g Arg	gag Glu 45	cac His	ttt Phe	tgg Trp	aat Asn	acg Thr 50	atg Met	agc Ser	ggc Gly	cac His	aat Asn 55	cca Pro	aaa Lys	gcg Ala	aaa Lys	677
aac Asn 60	tca Ser	act Thr	tgc Cys	cca Pro	tcc Ser 65	aaa Lys	aca Thr	ttt Phe	gaa Glu	tac Tyr 70	cgc Arg	ggt Gly	ctt L e u	cca Pro	tat Tyr 75	725
acg Thr	tat Tyr	atg Met	tgc Cys	agt Ser 80	gaa Glu	ttc Phe	agc Ser	aca Thr	aaa Lys 85	gca Ala	aaa Lys	tta Leu	aca Thr	gac Asp 90	tac Tyr	773
ttg Leu	acg Thr	ccg Pro	gtt Val 95	ttc Phe	aca Thr	aaa Lys	gac Asp	gcc Ala 100	att Ile	aaa Lys	aaa Lys	ggc Gly	ttg Leu 105	gaa Glu	aaa Lys	821
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tate	cctt	ct t	tttca	aaag	cg g	ctcg	tcate	c to	gtca	acct	ctt	gccg	cga (cccta	aaggaa	1246
aac	gccat	tat (gtgca	atag	cc g	gaage	cgtte	c tc		cctt e 15:		caag	gtc	gggg(cgtctc	1306

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tcctctaaat tgttcgtcca tatggcgatg tgttcgattt tcataaatct ccctcccatt	1486
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<211> 182

<212> PRT

<213> Bacillus licheniformis

<400> 70

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Asn Lys Gln Val Met Gln Leu Thr Leu Gln Ala Arg Glu His Phe Trp 35 40 45

Asn Thr Met Ser Gly His Asn Pro Lys Ala Lys Asn Ser Thr Cys Pro 50 60

Ser Lys Thr Phe Glu Tyr Arg Gly Leu Pro Tyr Thr Tyr Met Cys Ser 65 70 75

Glu Phe Ser Thr Lys Ala Lys Leu Thr Asp Tyr Leu Thr Pro Val Phe 85 95

Thr Lys Asp Ala Ile Lys Lys Gly Leu Glu Lys Tyr Asn Ile Ile Ser

Tyr Lys Gly Lys Met Ala Val Pro Val Gly Asp Gly Asp Asn Leu Leu 115 120 125

Gly Trp Asp Lys Ala Lys Ile Lys Leu Ile Ser Gln Lys Asn Asn Thr 130 135 140

Arg Thr Tyr Glu Phe Ser Val Pro Ala Leu Asp Gly Ser Val Thr Ala 145 150 155 160

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Gln Leu Asp Ala Ala Ile 180

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<211> 1621

<212> DNA

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<222> (271)..(1122)

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gcccagccgt tti	tttatgct gactcga	attt tctgaaac	at aatgaaaaga a	aatcgtatt 240
tgatgtaaag ct	tcttgaga ggatgaa	aaat gat gaa Asp Glu 1	aag aag cat cgg Lys Lys His Arg 5	cac ctt 294 His Leu
tta aat tta co Leu Asn Leu Ai 10	gg act gac aat t rg Thr Asp Asn I 15	tta ata ttc g Leu Ile Phe A	cg agt agg tta la Ser Arg Leu 20	agg gaa 342 Arg Glu
gcc gat tct to Ala Asp Ser Co 25	gt gat gca cgg a ys Asp Ala Arg A 30	arg Ser Phe G	aa ttg ccg cga llu Leu Pro Arg 5	gga att 390 Gly Ile 40
tgg gta tcg ag Trp Val Ser A	ga tct tta tta q rg Ser Leu Leu (45	gag ccg att c Glu Pro Ile L 50	tc tat cat cac eu Tyr His His	gcc ttc 438 Ala Phe 55
ccg tgc agg g Pro Cys Arg V	al Trp Pro Asp :	atc gaa aga a Ile Glu Arg <i>A</i> 65	at cgg gga cag Asn Arg Gly Gln 70	ttt gga 486 Phe Gly
act tgc ctg c Thr Cys Leu L 75	eu Leu His Met I	aaa ctg ctt g Lys Leu Leu <i>A</i> 80	at cat tta aat Asp His Leu Asn 85	atc aag 534 Ile Lys
aaa gtt cat g Lys Val His V 90	tg gtt gcg gtg al val Ala val 95	tca gcc ggc g Ser Ala Gly G	ggg cca agc gga Sly Pro Ser Gly 100	ata tgt 582 Ile Cys
ttt gca tcc a Phe Ala Ser L 105	aa tac tcg gaa ys Tyr Ser Glu 110	Arg Val Glu S	tcc tta att ttg Ser Leu Ile Leu L15	caa agc 630 Gln Ser 120
gct gtc aca a Ala Val Thr L	ag cag tgg ctg ys Gln Trp Leu 125	aca gcg aag c Thr Ala Lys A 130 Page	gat att gaa tat Asp Ile Glu Tyr 153	aaa gtt 678 Lys Val 135

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60

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ata Ile	tcg Ser	gcg Ala 155	ctt Leu	aac Asn	aat Asn	cga Arg	ttt Phe 160	ccg Pro	gaa Glu	tgg Trp	atc Ile	ttt Phe 165	aag Lys	aaa Lys	atg Met	774
cta Leu	tcc ser 170	tcc Ser	ttt Phe	act Thr	aca Thr	ctt Leu 175	cct Pro	gct Ala	gat Asp	cag Gln	gcg Ala 180	atg Met	ctg Leu	aaa Lys	gtc Val	822
acg Thr 185	gag Glu	gga Gly	gat Asp	att Ile	gaa Glu 190	gaa Glu	atg Met	aga Arg	aaa Lys	atg Met 195	aac Asn	aac Asn	aga Arg	cag G1n	cgt Arg 200	870
tca Ser	agt Ser	cga Arg	ggg Gly	ttc Phe 205	ttg Leu	ctt Leu	gat Asp	tta Leu	aaa Lys 210	aat Asn	ata Ile	gac Asp	gat Asp	tta Leu 215	tct Ser	918
ttc Phe	cat His	cat His	ttg Leu 220	aag Lys	gag Glu	att Ile	tct Ser	tgt Cys 225	ccg Pro	gta Val	tta Leu	att Ile	atg Met 230	cat His	tgc Cys	966
cga Arg	tat Tyr	gat Asp 235	cgt Arg	gtt Val	gtt Val	cca Pro	gcc Ala 240	gag Glu	cat His	gct Ala	ttt Phe	cat His 245	gca Ala	aaa Lys	aaa Lys	1014
	att Ile 250											tgg Trp				1062
att Ile 265	tgg Trp	ctg Leu	gga Gly	aca Thr	gag Glu 270	Gly	aaa Lys	tct Ser	gtc Val	tca Ser 275	GIN	aag Lys	gtc Val	atc Ile	agc ser 280	1110
	tta Leu				tcat	ctt	gatc	ataa	ga t	gaat	aaaa	t tt	tagg	atcg		1162
cago	cta	ccc	gcaa	atga	ag t	agtg	caat	t tt	ttaa	tcaa	gag	caga	atg	atct	ttccga	1222
acag	jaac	tga	tgaa	cgtc	gt a	caag	actt	g ca	aata	agat	gaa	tgag	aaa	tcct	ccctgg	1282
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tacg	gga	tga	atca	ttga	tt a	ccgt	tgcc	t gc	gatt	tccc	agg	9999	aga	gagt	tggtaa	1402
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ggca	agc	ggc	agct	tctt	cc t	aaac	tcgc	g at	tgaa	acga	tgc	ggct	atg	caat	atggtg	1582
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<210> 72

<211> 284

<212> PRT

<213> Bacillus licheniformis

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Ser Val Ser Gln Lys Val Ile Ser Phe Leu Lys Thr 275

<210> 73
<211> 1630
<212> DNA
<213> Bacillus licheniformis
<220>
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<223>

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ggt Gly	ctt Leu	tcc Ser	ttt Phe 160	ncc	gtc Val	tac Tyr	acg Thr	ctg Leu 165	aca Thr	agc Ser	aag Lys	aaa Lys	ctc Leu 170	ctg Leu	caa Gln	771
aag Lys	caa Gln	aag Lys 175	ccg Pro	gag Glu	gct Ala	gtc Val	aca Thr 180	ggc Gly	acc Thr	gta Val	ttc Phe	ttt Phe 185	tta Leu	agc Ser	gct Ala	819
gta Val	ttg Leu 190	ctt Leu	gcc Ala	ccg Pro	ttg Leu	ttg Leu 195	ttt Phe	ctg Leu	tac Tyr	gat Asp	ctc Leu 200	ggc Gly	tgg Trp	atc Ile	tca Ser	867
tcg ser 205	gtt Val	cag Gln	gga G1y	atg Met	gct Ala 210	gtc Val	agc Ser	ctc Leu	tat Tyr	atc Ile 215	ggg Gly	gtc Val	att Ile	gca Ala	acc Thr 220	915
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cgt	aato	aag	gttt	tcgg	at a	.aata	gatg	ıc cg	cggc	ggta	gtt	ttc	gta	aaga	agtat	g 1401
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															gatca	
															catat	
			gaga								•					1630

<210> 74

<211> 292

<212> PRT

<213> Bacillus licheniformis

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Thr Leu Ser Leu Ala Glu Pro Leu Thr Ala Ser Leu Leu Gly Thr Val 245 250 255 10294.000.sT25.txt Leu Val Arg Glu Ser Leu Pro Leu Val Ser Trp Ala Gly Ile Ala Leu 260 265 270 Leu Leu Gly Ile Phe Tyr Ile Ser Tyr Gln Pro Lys Lys Asp Lys 275 280 285 Ile Asn Ala Glu 290 75 <210> <211> 2140 <212> DNA Bacillus licheniformis <213> <220> <221> CDS (501)..(1637) <222> <223> <400> 60 agctgccggg gcaaatagag cgcgctggct tgcagataat gctgctggaa gagatactat 120 gaatacaagt gattatttag attttaatga agaaaacaaa aacaatgata atggaaaaga 180 ttacagtaat gcctacactg atatggactg tgaggcgatg actgaagata ttaatcattt 240 gaaatctgcc aatcctgagg tgtatcaaaa gctgcagaag atggacatta ccgctgcggc 300 gggatacaga acagaggata ctgtaagttt ttccccttac tataccgcaa gcggaaaaca 360 taaaataaac agtgatgata tcgtttcggt cgaaagtcaa cacggtgaca tattaggcga 420 tctcattgat aaaaagccag aaattgaagt aagaggttcc ggtgtaacca atcctggaca 480 tatttatgaa attgaagact ctgaatttgt tgacttgatt cgagaggtca acaaaaaga 533 581 tta ata gtg ttt cta ctg ctc gcc acc acc ggc tgc ggc aaa gat gat Leu Ile Val Phe Leu Leu Leu Ala Thr Thr Gly Cys Gly Lys Asp Asp 15 20 25 gtt cag gaa gcc atc tat aaa aaa ggc ttg ccc aaa gaa gac agt cca Val Gln Glu Ala Ile Tyr Lys Lys Gly Leu Pro Lys Glu Asp Ser Pro 30 35 629 gca ttt aga gaa ttt atg aga cat gaa ctt gat tta gcg aca gac gca Ala Phe Arg Glu Phe Met Arg His Glu Leu Asp Leu Ala Thr Asp Ala 45 50 677 act ctt agt tat caa aat agt aca tat acg att atg cgc agt gat aaa Thr Leu Ser Tyr Gln Asn Ser Thr Tyr Thr Ile Met Arg Ser Asp Lys 60 65 70 725

							-									
aag Lys	ggg Gly	cta Leu	cgg Arg	tac Tyr 80	tat Tyr	caa Gln	tat Tyr	aca Thr	gat Asp 85	caa Gln	gaa Glu	gta Val	gac Asp	gat Asp 90	ttt Phe	773
tac Tyr	agt Ser	ccc Pro	ttt Phe 95	ctt Leu	tcg Ser	gct Ala	aat Asn	aaa Lys 100	tat Tyr	cct Pro	gcg Ala	aca Thr	aaa Lys 105	tta Leu	tat Tyr	821
gat Asp	ttg Leu	aaa Lys 110	aca Thr	act Thr	gaa Glu	ttt Phe	tta Leu 115	act Thr	aaa Lys	gaa Glu	aaa Lys	ctt Leu 120	atc Ile	cac His	aat Asn	869
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agc Ser	atg Met	ctt Leu	ata Ile 175	caa Gln	gtg Val	gac Asp	gta Val	tat Tyr 180	gaa Glu	aaa Lys	ttt Phe	aaa Lys	aat Asn 185	ggt Gly	gac Asp	1061
ctt Leu	gga Gly	gac Asp 190	Arg	caa Gln	ata Ile	tat Tyr	tat Tyr 195	Leu	ttt Phe	tta Leu	aaa Lys	agt Ser 200	ASP	ctt Leu	tca Ser	1109
aaa Lys	tac Tyr 205	Arg	att Ile	gtt Val	aaa Lys	gaa Glu 210	gag Glu	gaa Glu	tta Leu	aat Asn	tca Ser 215	HIII.	att Ile	gag Glu	tct Ser	1157
ggg G l y 220	' Lys	ctg Leu	aag Lys	gaa Glu	tac Tyr 225	Leu	tcc Ser	gta Val	ttt Phe	cca Pro 230	ASI	gta Val	gcg Ala	aag Lys	gat Asp 235	1205
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aac Asn	aaa Lys	gtt Val	agg Arg 255	aaa Lys	atc Ile	aaa Lys	aac Asn	act Thr 260	ASP	att Ile	ctg Leu	ago Ser	aaa Lys 265	MOL	ggt	1301
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cct	gat Asp 285	G1y	ato Ile	caa Glr	cag Gln	ata Ile 290	GII	aca Thr	atg Met	gat Asp	aat Asr 295	ilyr	cta Leu	aaa Lys	gga Gly	1397
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tat Tyr	ttt Phe	aat Asr	aaa Lys 335	gat Asp	t tat Tyr	gta Val	gti Val	ttg Leu 340	I Туг)	. 116	e Sei	t tai	cat His 345	2 (1)	aag Lys	1541
									Pag	ge 10	50					

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Tyr Lys Lys Gly Leu Pro Lys Glu Asp Ser Pro Ala Phe Arg Glu Phe 35 40 45

Met Arg His Glu Leu Asp Leu Ala Thr Asp Ala Thr Leu Ser Tyr Gln 50 60

Asn Ser Thr Tyr Thr Ile Met Arg Ser Asp Lys Lys Gly Leu Arg Tyr 65 75 80

Tyr Gln Tyr Thr Asp Gln Glu Val Asp Asp Phe Tyr Ser Pro Phe Leu 85 90 95

Ser Ala Asn Lys Tyr Pro Ala Thr Lys Leu Tyr Asp Leu Lys Thr Thr 100 105 110

Glu Phe Leu Thr Lys Glu Lys Leu Ile His Asn Lys Leu Glu Tyr Asn 115 120 125 Leu Pro Glu Met Thr Leu Asp Lys Lys Asn Val Leu Lys Val Lys Thr 130 135 140 Lys Ser Gly Glu Lys Lys Ile Glu Phe Pro Ser Ala Lys Asp Lys Lys 145 150 160 Val His Leu Ala Leu Ala Ala Val Ser Lys Asp Ser Met Leu Ile Gln 165 170 175 Val Asp Val Tyr Glu Lys Phe Lys Asn Gly Asp Leu Gly Asp Arg Gln 180 185 190 Ile Tyr Tyr Leu Phe Leu Lys Ser Asp Leu Ser Lys Tyr Arg Ile Val Lys Glu Glu Glu Leu Asn Ser Thr Ile Glu Ser Gly Lys Leu Lys Glu 210 220 Tyr Leu Ser Val Phe Pro Asn Val Ala Lys Asp Gly Ala Tyr Arg Lys 225 230 235 Leu Phe Asp Lys Tyr Ile Phe Asp Glu Lys Lys Asn Lys Val Arg Lys 255 Ile Lys Asn Thr Asp Ile Leu Ser Lys Asp Gly Lys Tyr Val Tyr Ile 260 265 270 Asn Gly Ala Lys Glu Lys Glu Thr Asn Val Met Pro Asp Gly Ile Gln 285 285 Gln Ile Gln Thr Met Asp Asn Tyr Leu Lys Gly Asn Glu Lys Tyr Glu 290 295 300 Ala Gln Phe Lys Ile Asp Phe Lys Gln Ile Ala Lys Glu Met Asp Leu 305 310 315 Asn Ala Gly Asp Ala Arg Ile Ala Asn Ile His Tyr Phe Asn Lys Asp 325 330 335 Tyr Val Val Leu Tyr Ile Ser Tyr His Gly Lys Thr Ile Gly Thr Ala 340 350 Gly Ser Val Asn Val Leu Ile Asp Leu Gln Lys Asn Lys Gln Gln Pro 355 Thr Ala Tyr Leu Val Asp Leu Gly Ile Glu Ser 370

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cgc att atg aaa acc ttg aca gaa gaa gaa ttc cgg gca ggc tat cgc Arg Ile Met Lys Thr Leu Thr Glu Glu Glu Phe Arg Ala Gly Tyr Arg 30 35	629
aaa gcg cag ctc atc gat gtg cgc gag ccg aat gag tat gaa ggc ggc Lys Ala Gln Leu Ile Asp Val Arg Glu Pro Asn Glu Tyr Glu Gly Gly 45	677
cac att ttg ggt gcg aga aac att ccg ctt tca cag ctt aag caa aga His Ile Leu Gly Ala Arg Asn Ile Pro Leu Ser Gln Leu Lys Gln Arg 60 70 75	725
aaa agc gaa atc cgg cct gac aaa ccg gtt tac ctg tac tgc caa aac Lys Ser Glu Ile Arg Pro Asp Lys Pro Val Tyr Leu Tyr Cys Gln Asn 80 85 90	773
aac gtc aga agc gga agg gcc gcc caa acg ctc cgc aaa cac ggc tgt Asn Val Arg Ser Gly Arg Ala Ala Gln Thr Leu Arg Lys His Gly Cys 95 100 105	821
aag gag att tac aac ctg aaa ggc ggg ttc aaa aaa tgg ggc gga aaa Lys Glu Ile Tyr Asn Leu Lys Gly Gly Phe Lys Lys Trp Gly Gly Lys 110 120	869
Page 163	

att aaa acg aaa aat taataaccga agctgtctct gctatggaag gcttcagttg Ile Lys Thr Lys Asn 125	924
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Asp Val Arg Glu Pro Asn Glu Tyr Glu Gly Gly His Ile Leu Gly Ala 50 60

Arg Asn Ile Pro Leu Ser Gln Leu Lys Gln Arg Lys Ser Glu Ile Arg 65 75 80

Pro Asp Lys Pro Val Tyr Leu Tyr Cys Gln Asn Asn Val Arg Ser Gly 85 90

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•	581
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	629
cgt acg gca atg gat cag aaa gaa aac ggc cac gag cag gct gcc gaa Arg Thr Ala Met Asp Gln Lys Glu Asn Gly His Glu Gln Ala Ala Glu	QZ 3
30 35 40	677
aca gcc agg cag gaa gcc ggc tta aaa caa gtt gac agc gtg gag acg Thr Ala Arg Gln Glu Ala Gly Leu Lys Gln Val Asp Ser Val Glu Thr	0//
45 50 55	
ttt gtc ggt aaa gaa aag cag tac att gtt aca ggg gca gac aaa aaa Phe Val Gly Lys Glu Lys Gln Tyr Ile Val Thr Gly Ala Asp Lys Lys	725
Alle Adi dià rào dia cio di in ili alle in dia di di	
60 65 70 75	777
age dae aga ato tat off tog off cet off dae aga agg cag aga acg	773
60 65 70 73	773
ggc gac aaa atg tat gtt tgg gtg cct gct gac aaa aag cag aaa acg Gly Asp Lys Met Tyr Val Trp Val Pro Ala Asp Lys Lys Gln Lys Thr 80 85 90 ctt tac aaa aaa gca tca gcc ggc att acc ggc cgc cag gct gca aaa	821
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ggc gac aaa atg tat gtt tgg gtg cct gct gac aaa aag cag aaa acg Gly Asp Lys Met Tyr Val Trp Val Pro Ala Asp Lys Lys Gln Lys Thr 80 ctt tac aaa aaa gca tca gcc ggc att acc ggc cgc cag gct gca aaa Leu Tyr Lys Lys Ala Ser Ala Gly Ile Thr Gly Arg Gln Ala Ala Lys 95 gct gtt cag gat gag ggc ctg atg tct gag ctt aaa gag gtg cac ctt	
ggc gac aaa atg tat gtt tgg gtg cct gct gac aaa aag cag aaa acg Gly Asp Lys Met Tyr Val Trp Val Pro Ala Asp Lys Lys Gln Lys Thr 80 ctt tac aaa aaa gca tca gcc ggc att acc ggc cgc cag gct gca aaa Leu Tyr Lys Lys Ala Ser Ala Gly Ile Thr Gly Arg Gln Ala Ala Lys 95	821
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4 7 ~	Ara	ദിധ	:1v /	ısn i	/al !	Len I	1 <u>.</u>	0294 Trp (.000	.ST2	5.tx	t Tyr l	.eu /	Asn	Lys	
Ala											135					
gat Asp 140	ggg Gly	cag : Gln :	tac a Tyr S	ser l	tta a Leu s 145	agc 1 Ser 1	tat (Tyr '	gtg (Val /	asp i	ttt Phe 150	ata Ile	aac (Asn (gga Gly	Lys .	att Ile 155	965
cac His	aaa Lys	aat Asn	att a Ile :	acg (Thr 160	cct Pro	taga	cgaa	ac a	9999	gaaa	t cg	agtt	gaat			1013
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Gln	Lys	Glu 35	Asn	σΊу	His	Glu	G]n 40	Αla	Дlа	Glu	Thr	А]а 45	Arg	Gln	Glu	
۸٦a	G]y 50	Leu	Lys	Gln	٧a٦	Asp 55	Ser	val	G 1u	Thr	Phe 60	Val	Gly	Lys	Glu	
Lys 65	Gln	туг	Ile	٧a٦	Thr 70	Glу	Ala	Asp	Lys	Lys 75	GТу	Asp	Lys	Met	Tyr 80	
٧a٦	Тгр	Val	Pro	Ala 85	Asp	Lys	Lys	Gln	Lys 90	Thr	Leu	Туг	Lys	Lys 95	Ala	
ser	ΑΊа	Gly	Ile 100	Thr	GТу	Arg	G1n	A1a 105	Аlа	Lys	ΑΊа	Va1	G]n 110	Asp	Glu	

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115 120 125 Val Leu Leu Trp Glu Val Thr Tyr Leu Asn Lys Asp Gly Gln Tyr Ser 130 135 140 Leu Ser Tyr Val Asp Phe Ile Asn Gly Lys Ile His Lys Asn Ile Thr 145 150 160 Pro <210> 81 1993 <211> <212> DNA <213> Bacillus licheniformis <220> <221> **CDS** (501)..(1490) <222> <223> <400> 81 60 gggcttgccg gaatggaaac gatccttctg tttgcagcag gcattatttt aatcatttta gagatttttc tgccgggcgg aatagcaggg attgccggcc tgatcgcaat tgttgcgagc 120 ctctttttag cttcgggaag ctttaaggtc atggccgtct ccattttgat tgcaactgct 180 240 gtttcgatag cggcatccat tctattgaca agggtgttgg gtaagcgtat gaaatttttt 300 aaaaaattga tottaaccga otcgacaagc acagaaagcg gatacgtgto aaatgaaagc cggcgcgatt taattggcaa aatcggcgtc acgtatacac cgctcagacc gtccggaacg 360 420 gtcatcatcg acgatgaacg gcttgatgtt gtatctgaag gctcgttcac cgcaaaggat 480 aagaaagtga aagtggttaa agtggaaggc tcacgcattg ttgtgagaga attataaatt acatttttag gaggaatata atg gat ccg tca aca ctg ttt ctt tta ctt att
Met Asp Pro Ser Thr Leu Phe Leu Leu Ile
1 533 atc gca gcc gga atc atc cta cta gct gtc ttc ttt aca ttc gtc CCg Ile Ala Ala Gly Ile Ile Leu Leu Ala Val Phe Phe Thr Phe Val Pro 15 20 25 581 gtc atg ctg tgg atc tcg gct ttg gcc gct ggt gtt aaa atc agc att val Met Leu Trp Ile Ser Ala Leu Ala Ala Gly val Lys Ile Ser Ile 30 40 629 ttc aca ctg atc gga atg agg ctc cgc cgc gtc att cca aac cgc gtg Phe Thr Leu Ile Gly Met Arg Leu Arg Arg Val Ile Pro Asn Arg Val 45 50 55 677

Page 167

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gtc Val	aac Asn	gcg Ala	ctt Leu 95	atc Ile	gct Ala	gcc Ala	caa Gln	cgt Arg 100	gca Ala	aac Asn	att Ile	gaa Glu	ctt Leu 105	aca Thr	ttc Phe	821
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869

918

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Lys Asn Lys Gly Ile Phe Leu Lys Ser Gly Met Asp Gly Ile Ser Asn 145 150 160

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998

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Met Met Asp Tyr Ser Ser Phe Thr Glu Pro Lys Thr Ser Thr Thr Glu 65 75 80

Asp Gly Lys Ser Pro Asp Gln Ala Lys Asp Leu Ser Glu Ala Gln Lys 90 95 Page 208

Glu Lys Asp Lys Gln Ser Leu Lys Lys Ile Gln Glu Gln Val Asm Arg 100 105 110 Phe Ile Lys Glu Lys Asn Leu Gln Lys Gln Val Asn Thr Lys Leu Thr 115 120 125 Asp Glu Gly Leu Leu Leu Ser Ile Glu Asp Asn Ile Phe Phe Asp Ser 130 140 Gly Lys Ala Glu Ile Arg Gln Gln Asp Ile Pro Leu Ala Lys Glu Val 145 150 160 Ser Asp Leu Leu Val Leu Asn Pro Pro Arg Asn Ile Val Ile Ser Gly 165 170 His Thr Asp Asn Val Pro Ile Arg Asn Ser Gln Phe Lys Ser Asn Trp 180 185 190 His Leu Ser Val Met Arg Ala Val Asn Phe Met Gly Leu Leu Ile Glu 195 200 205 Asn Pro Lys Leu Asp Ala Lys Ile Phe Ser Ala Lys Gly Tyr Gly Glu 210 220 Phe Lys Pro Ile Ala Ser Asn Asp Thr Glu Glu Gly Arg Arg Lys Asn 225 235 240 Arg Arg Val Glu Ile Leu Ile Leu Pro Ile Gly Gln Glu Asn Leu Asn 245 250 255 Lys Lys Glu <210> 109 <211> 1735 <212> DNA

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100 105 110 Page 211	
·	

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gcg gca aac gaa aaa aaa ccg acg gtc acg agc cat acg tac aaa aac Ala Ala Asn Glu Lys Lys Pro Thr Val Thr Ser His Thr Tyr Lys Asn 35 40 45	683
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Ala	Ala	Asn 35	Glu	Lys	Lys	Pro	Thr 40	٧a٦	Thr	Ser	His	Thr 45	Tyr	Lys	Asn
Ile	Lys 50	Аlа	Leu	Lys	Туг	Pro 55	Gln	Val	ser	Asn	val 60	Ser	Pro	Lys	Ser
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Asp Met Asn Tyr Arg Gly Val Lys Ala Ala Leu Ala Gly Ala Tyr Leu 65 70 75 80

Arg Val Glu Gly Pro Lys Gly Lys Thr Thr Val Tyr Val Thr Asp Leu 85 90 95

Tyr Pro Glu Gly Ala Pro Gly Ala Leu Asp Leu Ser Pro Asn Ala Phe 100 105 110

Arg Glu Ile Gly Asp Met Lys Asp Gly Lys Ile Asp Ile Lys Trp Arg 115 120 125

Ile Val Lys Ala Pro Ile Thr Gly Asn Phe Thr Tyr Arg Ile Lys Glu 130 135

Gly Ser Ser Gln Trp Trp Ala Ala Ile Gln Val Arg Asn His Lys Tyr 145 150 160

Pro Val Met Lys Met Glu Tyr Tyr Lys Asp Gly Lys Trp Ile Asn Met 165 170 175

Glu Lys Thr Asp Tyr Asn His Phe Val Ser Thr Asn Leu Gly Thr Ser 180 185

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gca aac gtg ctc atg aga gtt ttc ggt tca gag gga aac aat aaa aat

Page 220

773

Ala Asn Val Leu Met Arg Val Phe Gly Ser Glu Gly Asn Asn Lys Asn	
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95 100 105	9.50
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110 115 120	917
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Thr Asp Gly Glu Glu Thr Cys Gly Gly Ash Pro Val Lys Val Ala Inr 160 165 170	
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175 180 185 185	
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90 Ser Cys Asn Ala Ile Arg Gly Val Tyr Gly Phe Gln Thr Tyr Asp Glu 100 105 110 Gln Ser Phe Arg Asn Ser Leu Asn Gly Ile Gly Pro Thr Gly Trp Thr 115 120 125 Pro Ile Ala Asn Ala Leu Gln Asp Ala Lys Asn Ala Leu Asp Gln Leu 130 140 Asp Asn Asn Gly Lys Asn Val Val Tyr Leu Leu Thr Asp Gly Glu Glu 145 150 155 Thr Cys Gly Gly Asn Pro Val Lys Val Ala Thr Glu Leu Arg Lys Ser 165 170 175 Asn Ala Val Val Asn Val Ile Gly Phe Asp Tyr Glu Gly Asp Phe His 180 185 190 Gly Gln Leu Thr Ser Ile Ala Ala Gly Gly Gly Glu Tyr Phe Gln
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Bacillus licheniformis

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45

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Glu Ala Val Tyr Asp Lys Gln Glu Arg Glu Glu Ala Ser Ile Lys Asp 65 70 75 80

Tyr Leu Asn Gly Ala Asp Arg Glu Gly Glu Glu Ala Leu Asn Glu Met 85 90 95

Lys Met Val Leu Ser Glu Leu Ser Ile Ala Lys Ser Asp Pro Glu Gln 100 100 110

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Thr Val Lys Ser Phe His Tyr Leu Thr Val Asp Gly Lys Asn Val Asp 50 Page 226

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Met Val Leu Pro Lys Gln Asn Lys Asn Gly Asp Leu Leu Ala Tyr Gly 85 90 95	
Phe Thr Ser Lys Val Thr Leu Glu Ala Phe Ile Ala Lys Asp Lys Gln 100 105 110	
Arg Leu Glu Lys Gln Phe Lys Pro Ser Ala Ser Gly Pro Cys Cys Thr 115 120 125	
Asp Phe Tyr Glu Tyr Lys Asn Lys Gly Gly Gln Tyr Ile Tyr Trp Arg 130 135	
Asp Gly Phe Lys Asn Leu Pro Ser Ser Trp Asn Asp Arg Ile Ser Ser 145 150 155 160	
Leu Ser Thr Ala Ser Pro Ser Ser Ser Tyr Ser Thr Thr Leu Trp Glu 165 170 175	
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Gly Ile Glu Asn Lys Val Glu Ala Ala Arg Thr Leu Glu Asp Phe Lys 50 60

Ala Ala Tyr Lys Gly Trp Gln Leu Ile Asp Gln Lys Lys Gly Phe Ile 65 70 75 80

Leu Phe Arg Lys Gln Val Asp Asp Ile Ser Pro Leu Ser Lys Thr Asn 85 90 95

Gly Tyr Ile Gly Val Thr Glu Asp Gly Val Ile Ser Thr Phe His Gly 100 105

Arg Pro Gly Ile Leu Ser Glu Pro Ile Gln Ser Phe Phe Gln Ile Asp 120 125

Ile Lys Arg Leu Glu Ser Arg Met Ala Asp Asp Leu Arg Lys Gly Ile 130 140

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Gln Ser Gly Val Lys Leu Val Thr Val Asn Leu Leu Asn Ala Glu Lys 85 90 95	
Asn Glu Gln Lys Val Lys Gln Phe Ile Lys Ala Asn Lys Leu Thr Phe 100 105 110	
Pro Ile Val Phe Asp Lys Lys Gly Glu Met Met Lys Ala Tyr Lys Val 115 120 125	
Mot The Tie Dee The The Dhe Dhe ter Civiles Civiles Civiles	
Met Thr Ile Pro Thr Thr Phe Phe Asn Glu Lys Gly Glu Leu Glu 130 140	
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gat aca tat ttg gaa aaa ttg gtc gta aag ttt ggt gat gaa atc gtt Page 232	773

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540

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660

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Tyr Gln Tyr Pro Ser Glu Gly Gly Thr Trp Arg Tyr Gly Phe Val Asn 50 60

Ala Gly Leu Arg Ser Glu Tyr Asn His Pro Thr Lys Val His Gly Ser 65 70 75 80

Thr Val Gln Lys Leu Ile Asp Gly Lys Val Asp Lys Thr Asn Arg Ser 85 90 95 Page 239

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Leu Lys Lys Lys Met Thr Val Leu Glu Glu Glu Leu Leu Asp Ser Asn $50 \hspace{1.5cm} 55 \hspace{1.5cm} 60$

Val Val Val Arg Arg Pro Asn Ala Gly Ile Ser Gln His Ile Ala Lys 65 70 75 80

Gln Ile Leu Ser Lys Tyr Gln Asn Gly Met Ser Val Asp Ala Ile Ala 85 90 95

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Glu Lys Glu Gly Leu Lys Leu Glu Ile Val Lys Tyr Ser Asp Tyr Val 50 60

Gln Pro Asn Gln Ala Leu Ala Ser Gly Asp Ile Asp Arg Gln Arg Phe 65 70 75 80

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PRT

Bacillus licheniformis

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His Ser Thr Ala Gln Ile Val Tyr Ile Leu Pro Pro Glu Gln Ala Phe 50 60

The Asp Leu Phe Ser Asp Pro Thr Gly Arg Phe Val Phe His Pro Arg 75 75 80

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Ser Lys Glu Glu Gly Met Gln Gly 175 180

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45

His Ile His Asp Gln Ser Glu Arg Arg Phe Ile Ile Glu Lys Glu Thr

Glu Met Ile Gly Leu Val Glu Leu Val Glu Ile Asp Tyr Ile His Arg 65 70 75 80

Arg Ala Glu Phe Gln Ile Ile Ile Asp Pro Glu His Gln Gly Asn Gly 90 95

Tyr Ser Ser Ser Ala Thr Tyr Leu Ala Met Asn Tyr Ala Phe Ser Val 100 110

Leu Asn Leu His Lys Leu Tyr Leu Ile Val Asp Glu Asp Asn Ala Lys 115 120 125

Ala Ile His Leu Tyr Lys Lys Ala Gly Phe Thr Ile Glu Ser Glu Leu 130 135

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Page 250

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tac ggc tac acc gtt gtt cat tat agt att aac tcg gat gac tgg acg Tyr Gly Tyr Thr Val Val His Tyr Ser Ile Asn Ser Asp Asp Trp Thr 175 180 185	1060
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185 Ile Val Gln Asn Val Asn Gly Thr Val Asn Ala Gly Asp Ile Val Leu 195 200 205 Phe His Ala Ser Asp Ser Ala Lys Gln Thr Lys Glu Ala Leu Pro Glu 210 215 Ile Val His His Leu Arg Ser Lys Gly Leu Lys Asn Val Thr Val Ser 225 230 240

Page 252

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Arg Ile Pro Thr Phe Glu Glu Val Leu Asp Arg Tyr Lys Gly Lys Val 130 135 140

Gly Met Leu Ile Glu Leu Lys Glu Pro Ala Arg Tyr Pro Gly Ile Glu 145 150 155 160

Gly Lys Val Ser Ala Ala Leu Lys Glu Arg Arg Met Asp Lys Pro Lys 165 170 175

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Ala Ser Asp Asp Ile Gln Glu Met Met Asp Ala Gly Ser Ser Ser Asn 50 Page 258

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Lys Ala Val Ile Met Asn Ile Lys Ser Pro Glu His Pro Arg Gly Ser	
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gtg aac aac atg aaa atc ggc gaa tgg atc gag aca ttt aag ccg Page 260	674

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Thr Ile Phe Asn Ala Glu Gly Cys Thr Leu Asn Ile Lys Leu Gln Arg 65 70 80

Ile Thr Leu Thr Gly His Ala Val Thr Leu Ser Glu Lys Glu Tyr Thr 85 90 95

Gly Asn His Leu His Leu Ser Ala Ala Asp Lys Val Ser Gly Ser Pro 100 105

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Cag gaa atc gag aag ctg gaa acg ctg gca gcc aaa ttg ggc gcc gaa Gln Glu Ile Glu Lys Leu Glu Thr Leu Ala Ala Lys Leu Gly Ala Glu 45 50 55	677
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Leu Glu Thr Leu Ala Ala Lys Leu Gly Ala Glu Arg Glu Thr Asp Phe 50 Fage 265

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Page 270	

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60

120 176

224

272

320

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Ala Phe Thr Glu Gln Asp Pro Asp Gln Asn Gly Lys Asp Asp Thr Phe 180 185 190 Page 275

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1539

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Pro Glu Val Arg Ala Ile His Gln Glu Phe Asn Lys Tyr Ser Pro Glu 195 200 205 Page 282

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Page 288

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Page 290

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Page 292

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Glu Thr Asn Val Lys Thr Leu Glu Phe Leu Lys Thr Leu Ile Glu Lys 225 230 235 240

Page 295

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Gly Asn Ile Gln Thr Asp Ser Leu Pro Ser Ala Tyr Ala Lys Trp Arg 100 105 110 Page 298

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724

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Ala Asn Gly Ile Lys Asp Ala Thr Phe Phe Leu Ser Ala Ser Trp Ala 85 90 95

Glu Arg His Pro Asp Val Val Glu Arg Ile Arg Lys Asp Gly His Gln 100 105

Ile Gly Ser Met Gly Tyr Ala Tyr Lys Asn Tyr Ser Gln Met Lys Lys 115 120 125

Ser Glu Ile Lys Lys Asp Leu Ala Lys Ala Arg His Ser Phe Gln Lys 130 140

Leu Gly Leu Asp Asp Leu Thr Leu Leu Arg Pro Pro Thr Gly Gln Phe 145 150 155 160

Asn Lys Asp Val Leu Asp Val Ala Lys Gln Tyr Gly Tyr Thr Val Val 165 170 175

His Tyr Ser Ile Asn Ser Asp Asp Trp Thr Asn Pro Gly Val Gln Lys 180 185 190

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Phe His Ala Ser Asp Ser Ala Lys Gln Thr Lys Glu Ala Leu Pro Glu 210 215 220

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Page 302

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act Thr	gcc Ala	aaa Lys	gaa Glu 175	ctg Leu	acg Thr	gtt Val	aca Thr	gca Ala 180	acg Thr	gca Ala	tac Tyr	act Thr	gcc Ala 185	aat Asn	gac Asp	1061
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<213> Bacillus licheniformis

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tac aac atc att tct tat aaa gga aaa atg gcc gtg cct gtc g Tyr Asn Ile Ile Ser Tyr Lys Gly Lys Met Ala Val Pro Val 6 110	
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Caa aaa aac aat acc cgc act tat gaa ttt tcc gta ccg gca t Gln Lys Asn Asn Thr Arg Thr Tyr Glu Phe Ser Val Pro Ala L 140 150	ttg gat 965 Leu Asp 155
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<213> Bacillus licheniformis

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Asn Lys Gln Val Met Gln Leu Thr Leu Gln Ala Arg Glu His Phe Trp 35 40 45

Asn Thr Met Ser Gly His Asn Pro Lys Ala Lys Asn Ser Thr Cys Pro 50 60

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tatagtgtac acaatttctt cttaattttt gtataaacac tgttgacaag gaaaaaatag Page 307

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60

120

180

240

300

360

420

480

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aca gcc cgc atg tct aag tgg gaa gaa aaa gcg gtt gag gaa gca aaa Thr Ala Arg Met Ser Lys Trp Glu Glu Lys Ala Val Glu Glu Ala Lys 30 35 40	629
aag aga tat ccg gaa gca gaa gtg cgc ctc acg aaa aaa gta tgg gat Lys Arg Tyr Pro Glu Ala Glu Val Arg Leu Thr Lys Lys Val Trp Asp 45 50 55	677
cga aag cgg gcc gat gaa gcg gtc aaa caa tac cat gtc aca ttg agt Arg Lys Arg Ala Asp Glu Ala Val Lys Gln Tyr His Val Thr Leu Ser 60 65 70 75	725
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gcg aca cac aaa att aac aaa gtc gtc gtt gtg gaa gaa tat aaa Ala Thr His Lys Ile Asn Lys Val Val Val Glu Glu Tyr Lys 95 100 105	818
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gtc Val 55	atc Ile	tta Leu	atc Ile	ggg Gly	tta Leu 60	acg Thr	tac Tyr	gcg Ala	gag Glu	ctg Leu 65	tct Ser	tct Ser	gcc Ala	atc Ile	cct Pro 70	727
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									rag	e 31	U					

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The His Pro Lyš Tyr Lys Thr Pro Tyr Asn Ala Ile Leu Phe Leu Gly gcg Ctg gcg ttt ttt gcc ccg ctg ctc gga cgc cct gcc ctt gtt tgg Ala Leu Ala Phe Phe Ala Pro Leu Ceu Gly Arg Pro Ala Leu Val Trp 355 atc gtc aat gca ggg gga aca ggt att ata gtc gga tat ttg atc gtc Ile Val Ala Asn Ala Gly Gly Thr Gly Ile Ile Val Gly Tyr Leu Ile Val 360 Asn Ala Gly Gly Thr Gly Ile Val Gly Tyr Leu Ile Val 360 atc gatt gca ttc atg aag agg aaa aca gag ccg gat tta cac agg ser Ile Ala Phe Met Lys teu Arg Lys Thr Gly Ile Ser Ala Ile Leu Ang Asn Lys Trp Lys Thr Thr Gly Ile Ser Ala Ile Leu Ang Lys Trp Lys Thr Gly Ile Ser Ala Ile Leu Ang Lys Thr Gly Ile Ser Ala Ile Leu Ang Gly Tyr Leu Ile Val 380 ccg tat aaa atc aaat aag tgg aaa aca acg ggt ata tcg gct atc cec Tyr Lys Ile Asn Lys Trp Lys Thr Thr Gly Ile Ser Ala Ile Leu Ang Gly Tyr Leu Pro Gly Met Pro Ala Ala Ala Ala Ala Phe Phe Leu Ala Phe Tyr Leu Pro Gly Met Pro Ala Ala Ala Ala Ala Phe Pro Tyr Glu Trp Leu Ile Ala Fro Tyr Lys Ile Asn Lys Trp Lys Thr Ala Gly Trp Thr Leu Ile Thr Trp Pro Tyr Glu Trp Leu Ile Leu Ala Gly Trp Ala Gly Trp Thr Leu Ile Ala Arg Ser Ile Ala	ctg Leu	ttt Phe	gca Ala	atg Met	tcg Ser 315	gaa Glu	aag Lys	ggc Gly	atg Met	gtg Val 320	ccg Pro	aaa Lys	tgg Trp	ttc Phe	ggc Gly 325	ttc Phe	1495
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Page 318

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Page 326

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Asp Asp Ile Ile Asp Lys Ile Asp Leu Asn Gly Glu Gln Val Val 50 55 60 Page 327

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Lys Arg Glu Asn Gly Asn Tyr Gln Trp Tyr Arg Asp Leu Asn Tyr Ala 85 90 95	
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Pro Phe Thr Thr Pro Lys Gly Arg Lys Tyr Thr Leu Tyr Thr Gly Asp 115 120 125	
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1397

1445

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Gly Phe Glu Asp Ala Ala Gln Leu Phe Asn Val Ser Val Glu Tyr Arg 65 75 80

Gly Ala Ala His Tyr Asp Val His Glu Gln Thr Thr Val Leu Glu Gln 90 95
Page 330

Val Ile Ala Lys Lys Pro Ala Gly Ile Ala Val Ser Ala Ile Asn Pro 100 105 110 Lys Ala Leu Asn Pro Val Ile Asp Lys Ala His Glu Gln Gly Ile Pro 115 120 125 Ile Val Leu Phe Asp Ser Asp Ala Pro Leu Ser Lys Ala Ser Thr Tyr 130 140 Ile Gly Thr Asn Asn Met Glu Ala Gly Ala Val Ala Ala Arg Arg Met 145 150 155 160 Ala Glu Phe Leu Asn Gly Lys Gly Glu Thr Ala Val Ile Thr Gln Pro 165 170 175 Gln Gln Tyr Asn His Gln Glu Arg Thr Lys Gly Phe Glu Gln Thr Ile 180 185 Lys Gln Lys Tyr Pro Asn Met Lys Val Ala Ala Val Leu Asp Gly Lys 195 200 205 Gly Asp Glu Leu Thr Ser Lys Lys Glu Ala Ala Lys Ile Leu Glu Glu 210 220 Asn Pro Ser Ile Lys Gly Ile Phe Thr Thr Glu Ala Asn Gly Ala Ser 225 230 235 Gly Val Ala Arg Ala Val Lys Glu Ala Gly Leu Glu Gly Glu Val Cys 245 250 Ile Ile Gly Phe Asp Lys Asp Lys Lys Thr Leu Asp Gly Ile Lys Asn 260 265 Gly Ser Ile Ser Ala Thr Met Ser Gln Asp Thr Trp Gln Met Gly Tyr 275 280 285 Trp Ser Leu His Met Leu Phe Phe Ser Asn His His Leu Lys His Glu 290 300 Arg Pro Leu Pro Ala Ala Ile Asp Thr Gly Ile Thr Ile Ile Thr Lys 305 310 315 Glu Asn Val Ala Ala Tyr Tyr Ala Asn Asp 325 330

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160 165 170
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                                                                                               1061
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190 195 200
                                                                                               1109
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205 210 215
                                                                                               1157
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Thr Asn Leu Leu Gln Gln Tyr Asp Ala Phe Tyr Leu Gly Asn Thr Lys 50 60

Glu Lys Thr Ile Tyr Leu Thr Phe Asp Asn Gly Tyr Glu Asn Gly Tyr 65 70 75 80 Thr Pro Gln Val Leu Asp Val Leu Lys Lys Gln Asn Val Lys Ala Ala 85 90 95 Phe Phe Val Thr Gly His Phe Val Lys Asp Gln Pro Glu Leu Ile Lys 100 105 Arg Met Ala Glu Glu Gly His Ile Ile Gly Asn His Ser Tyr His His 115 120 125 Pro Asp Leu Thr Thr Lys Thr Ser Arg Val Ile Gln Glu Glu Leu Glu 130 140 Ser Val Asp Glu Glu Val Tyr Lys Ile Thr Gly Glu Lys Asn Asn Leu 145 155 160 Tyr Leu Arg Pro Pro Arg Gly Ile Phe Ser Glu Arg Val Leu Glu Glu 175 Thr Lys Lys Leu Gly Tyr Gln Thr Val Phe Trp Ser Val Ala Phe Val 180 185 190 Asp Trp Lys Ile Asn Ala Gln Lys Gly Trp Arg Tyr Ala Tyr Asp Asn 195 200 205 Met Met Lys Gln Ala His Pro Gly Ala Ile Tyr Leu Leu His Thr Val 210 220 Phe Arg Arg Ser Pro Thr 225 230 <210> 199 <211> 4041 <212> DNA <213> **Bacillus licheniformis** <220> <221> CDS <222> (501)..(3641) <223>

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Page 335

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	ata Ile															1829
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